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Induction and expression of *βig-h3* in pancreatic cancer cells

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Abstract

βig-h3 (TGFBI, keratoepithelin) was first identified as a transforming growth factor-β1 (TGF-β1)-inducible gene in a human lung adenocarcinoma cell line. It encodes for a secreted extracellular matrix (ECM) protein, which is thought to act on cell attachment and ECM composition. Mutations of the *βig-h3* gene are involved in several corneal dystrophies. Pancreatic cancers display multiple alterations in the TGF-β signaling pathway and in TGF-β response genes, such as overexpression of all three TGF-β isoforms and Smad4 mutations. In this report, we determined that *βig-h3* mRNA levels were induced by TGF-β1 in two out of five examined pancreatic cancer cell lines (CAPAN-1, PANC-1). In CAPAN-1 cells, which harbor a Smad4 mutation, *βig-h3* but not PAI-1 was induced by TGF-β1, whereas in PANC-1 cells that express wild-type Smad4, TGF-β1 induced both PAI-1 and *βig-h3*. In human pancreatic tissues, there was a 32.4-fold increase in *βig-h3* mRNA levels in pancreatic cancers in comparison to normal control tissues. In situ hybridization analysis revealed that *βig-h3* mRNA was expressed mainly in the cancer cells within the pancreatic tumor mass. These findings suggest that *βig-h3* is induced by TGF-βs in pancreatic cancer cells even in the presence of Smad4 mutations, which might explain, in part, the increased *βig-h3* mRNA levels observed in pancreatic cancer cells in vivo.

Keywords: *βig-h3*; TGF-β; SMAD4; Pancreatic cancer; PAI-1

1. Introduction

βig-h3 (TGFBI, keratoepithelin) is a gene that was first discovered by differential expression analysis of a transforming growth factor-β1 (TGF-β1)-treated human lung adenocarcinoma cell line [1]. It encodes a 68-kDa protein with an amino-terminal secretory sequence and a carboxy terminal sequence that can serve as a ligand recognition site for several integrins [1]. Subsequent analysis revealed that *βig-h3* is strongly induced by TGF-β1 in several cell lines, including human melanoma cells, human mammary epithelial cells, human keratinocytes, and human fibroblasts [2,3]. Expression of *βig-h3* has also been observed in corneal epithelial cells [4], skin fibroblasts [2], endothelial cells [5], in the juxtaglomerular apparatus and the pars recta of the proximal tubules in the kidney [6], in bladder smooth muscle cells, and in bladder urothelium [7].

Further functional analysis has revealed that *βig-h3* is secreted into the extracellular matrix (ECM), and that it may function as an extracellular attachment protein, thus influencing cell attachment and migration [2]. These initial results led to further investigations regarding the role of *βig-h3* in human diseases. For example, in coronary heart disease, restenotic coronary lesions showed high levels of *βig-h3* in areas of dense fibrous connective tissue. *βig-h3* was present in the cytoplasm of plaque macrophages as well as smooth muscle and endothelial cells. [5]. Most of the research has focused on the role of *βig-h3* in corneal dystrophy [8]. Thus, *βig-h3* mutations have been detected in six different autosomal dominant corneal dystrophies, such as Fuchs' dystrophy, Avellino corneal dystrophy,

Abbreviations: ECM, extracellular matrix; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary

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Sardinian Reis Bucklers corneal dystrophy, and others, and the excessive *βig-h3* content in these corneas suggests that the mutated gene product is a fundamental constituent of the characteristic corneal accumulations [8–14].

Our current knowledge of the potential role of *βig-h3* in malignant processes is limited to data from cultured cell lines. For example, SV40-transformed human fibroblasts exhibit down-regulation of *βig-h3* expression in comparison to normal fibroblasts, suggesting that *βig-h3* expression is reduced during the malignant transformation of this cell type [15]. This is supported by experiments with Chinese hamster ovary (CHO) cells, in which transfection of a *βig-h3* expression plasmid led to a marked decrease in the ability of these cells to form tumors in nude mice [3].

In epithelial cells, TGF-βs usually exert strong growth inhibitory effects [16]. However, all three TGF-β ligands are overexpressed in pancreatic cancer, and that overexpression is associated with decreased patient survival following tumor resection [17]. Furthermore, expression analysis of the receptors has revealed that the type II and type I TGF-β signaling receptors are also present in pancreatic cancer [18,19], raising the possibility that TGF-βs may act in an autocrine manner in this malignancy to promote tumor growth. However, these cancer cells have lost their ability to respond to the growth suppressive effects of TGF-βs because of Smad4 mutations [20], decreased TGF-β receptor type I (TβRI) expression in a subgroup of patients [21,22] and overexpression of the TGF-β signaling inhibitors Smad6 and Smad7 [23,24]. Therefore, it has been difficult to demonstrate convincingly that TGF-βs act in an autocrine manner in pancreatic cancer. Since *βig-h3* is a downstream target of TGF-β1, in the present study, we examined the effects of TGF-β1 on *βig-h3* expression in cultured pancreatic cancer cells in relation to their Smad4 status, and determined whether *βig-h3* is present in human pancreatic tissues. We now show that TGF-β1 is able to induce *βig-h3* in pancreatic cancer cell lines, irrespective of whether Smad4 is mutated or not, and that *βig-h3* is overexpressed in pancreatic cancer cells in vivo.

2. Methods

2.1. Cell culture

Human pancreatic cancer cells were routinely grown in DMEM (PANC-1, MIA-PaCa-2) or RPMI (ASPC-1, CAPAN-1, T3M4) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were maintained at 37 °C in a humid chamber with 5% CO_2 and 95% air atmosphere. For TGF-β1 experiments, subconfluent cells were incubated overnight in serum-free medium (containing 0.1% BSA, 5 μg/ml transferin, 5 ng/ml sodium selenite, and antibiotics), and subsequently incubated with 1 nM of TGF-β1 for the indicated time. Cells were then harvested for RNA extraction. ASPC-1, CAPAN-1, PANC-1, and MIA-PaCa-2 human pancreatic cell lines were obtained from ATCC (Rockville, MD, USA). T3M4 human pancreatic cell lines were a gift from Dr. R.S. Metzgar (Durham, NC, USA). TGF-β1 was a gift from Genentech, Inc. (South San Francisco, CA, USA).

2.2. Probe synthesis for Northern blot analysis

A fragment of human *βig-h3* cDNA was amplified by RT-PCR and subcloned into the pGEM-T Easy vector (Promega Biotechnology, Madison, WI, USA) according to the manufacturer's instructions. The identity of the cDNA fragments was confirmed by sequence analysis using the dye terminator method (ABI 373A, Perkin Elmer, Rot Kreuz, Switzerland). The mouse 7S cDNA probe that cross-hybridizes with human 7S and the human PAI-1 cDNA probe were generated as reported previously [24]. For Northern blot analysis, the probes were radiolabeled with ^{32}P-dCTP (Du Pont International, Regensdorf, Switzerland) using a random primer labeling system (Roche Diagnostic Ltd., Rotkreuz, Switzerland). For in situ hybridization, digoxigenin-labeled *βig-h3* cRNA sense and antisense probes were generated using the Ribomax System (Promega Biotechnology) and the appropriate polymerases [24].

2.3. Northern blot analysis

Following electrophoresis of total RNA in a 1.2% agarose/1.8 M formaldehyde gel, RNA was electrotransferred onto nylon membranes (Gene Screen, Du Pont, Boston, MA, USA) and cross-linked by UV irradiation. The filters were then prehybridized for 5 h at 42 °C and hybridized for 20 h at 42 °C in the presence of the radiolabeled cDNA probes for *βig-h3*. Blots were then rinsed twice with 2 × SSC at 50 °C and washed twice with 0.2 × SSC/2% SDS at 55 °C for 10 min. All blots were exposed

Table 1
Summary of TGF-β receptor expression, Smad4 mutations and effects of TGF-β1 on cell growth and PAI-1 and *βig-h3* expression in pancreatic cancer cell lines

	ASPC-1	CAPAN-1	MIA-PaCa-2	PANC-1	T3M4
TβRI [22]	+	++	+	++	+
TβRII [22]	+	++	–	+	+++
Smad4 [26]	mutated	mutated	wild type	wild type	wild type
Growth [22]	0	0	0	–	0
PAI-1 induction	0	0	0	+	0
βig-h3 induction	0	+	0	+	0

Expression of TβRI, TβRII as described previously [22]. Symbols: low (+), moderate (++), high (+++) expression by Northern blotting. Growth responsiveness to TGF-β1 as described previously [22]. Symbols: no TGF-β1 responsiveness (0); weak to moderate (–) growth inhibition by TGF-β1. Induction of PAI-1 and *βig-h3* mRNA expression was determined by Northern blot analysis. Symbols: no induction (0), moderate to strong induction (+).

at − 80 °C to Kodak BioMax films with Kodak intensifying screens, and the intensity of the radiographic bands was quantified by video image analysis, using the Image-Pro plus software (Media Cybernetics, Silver Spring, MD, USA). To verify equivalent RNA loading on Northern blot membranes, filters were rehybridized with the 7S cDNA probe, as reported previously [24].

2.4. Patients and tissue collection

Normal pancreatic tissue samples were obtained through an organ donor program from 11 individuals who were free of any apparent disease. The median age of the organ donors was 34.8 years, with a range of 14 to 52 years. Pancreatic cancer tissue samples were obtained from 27 pancreatic cancer patients (9 female and 18 male, median age 69.6 years) undergoing pancreatic resection. Freshly removed tissue samples were fixed in paraformaldehyde solution for 12 to 24 h and paraffin-embedded for in situ hybridization. Tissue samples for RNA extraction were immediately snap frozen in liquid nitrogen upon surgical removal in the operating room and maintained at − 80 °C until use. All studies were approved by the Human Subjects Committee of the University of Bern.

2.5. In situ hybridization

The tissue sections (4 μm) were deparaffinized, dehydrated, and incubated in 0.2 M HCl for 20 min. The sections were treated with proteinase K (50 μg/ml) for 15 min at 37 °C. Following post-fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, the samples were prehybridized at 60 °C for 2 h in 50% formamide (v/v), 4 × SSC, 2 × Denhardt's solution and 250 μg/ml RNA. Hybridization was performed overnight at 60 °C in 50% (v/v) formamide, 4 × SSC, 2 × Denhardt's solution, 500 μg/ml RNA and 10% dextran sulfate (w/v). The final concentrations of the digoxigenin-labeled probes were approxi-

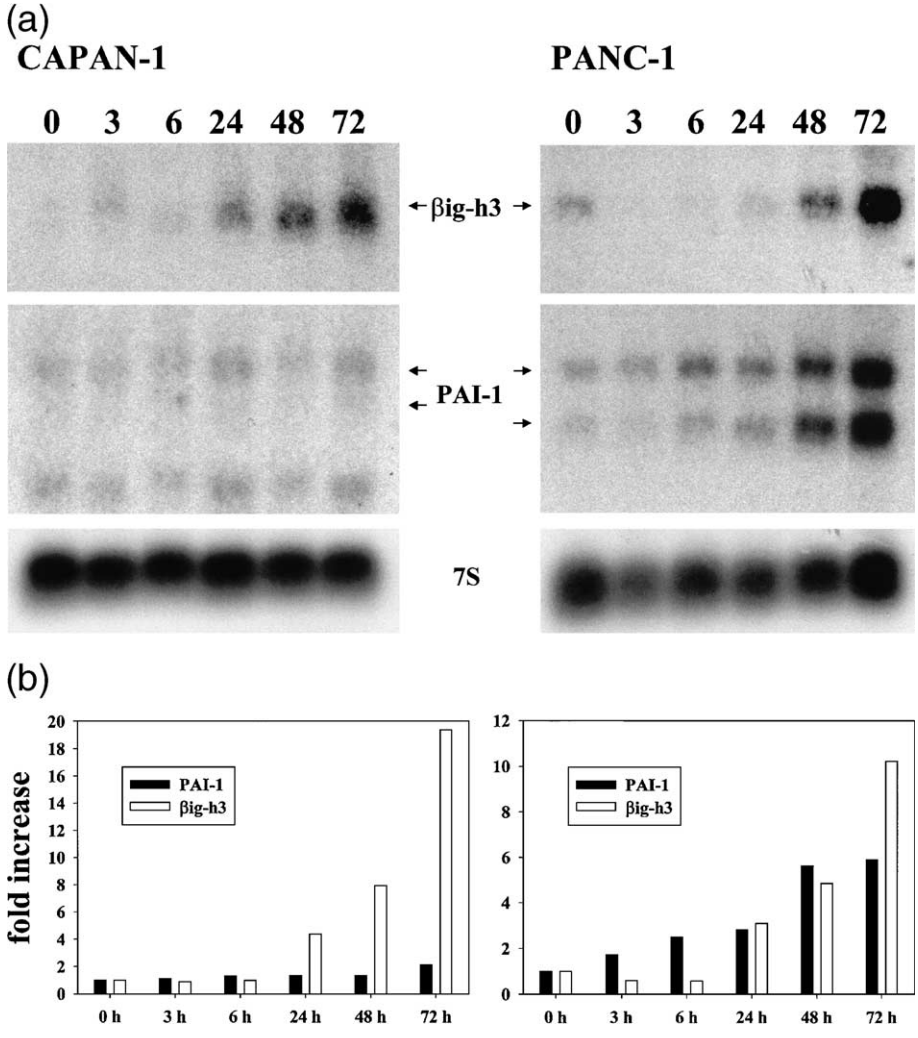

Fig. 1. (a) Effects of TGF-β1 on PAI-1 and *βig-h3* levels. Cells were serum-starved for 12 h and incubated in the absence (0 h) or presence of 1 nM TGF-β1 for the indicated time. Northern blot analysis of total RNA (20 μg/sample) was performed with ^{32}P-labeled PAI-1, *βig-h3* and 7S cDNA probes. (b) Bar graph of two experiments. The $OD_{\beta ig\text{-}h3}/OD_{7S}$ and $OD_{PAI\text{-}1}/OD_{7S}$ at 0 h were set to 1.

mately 0.5 ng/μl. After hybridization, the sections were washed and treated with RNase. The samples were then incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (1:500). For color reaction, 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium (Sigma, Buchs, Switzerland) were used. For control experiments, the slides were incubated with RNase or with the corresponding sense probes. Pretreatment of the slides with RNase abolished the hybridization signals produced by the antisense probe. Furthermore, incubation with the sense probe failed to produce specific in situ hybridization signals.

3. Results and discussion

Cultured pancreatic cancer cell lines are usually resistant to the growth inhibitory effects mediated by TGF-β1 [22,25]. In the present study, we utilized five pancreatic cancer cell lines (ASPC-1, CAPAN-1, MIA-PaCa-2, PANC-1, and T3M4) to assess whether TGF-β1 induces *βig-h3* expression in these cells (Table 1). We have previously shown that of those five pancreatic cancer cell lines, only PANC-1 is growth inhibited by TGF-β1, and that this cell line expresses TβRI and TβRII and possesses a normal Smad4 gene [22,26]. ASPC-1 and CAPAN-1 cells are resistant towards the growth inhibitory effects of TGF-β1 due to Smad4 mutations [26], T3M4 cells are resistant because they underexpress TβRI [21], whereas MIA-PaCa-2 cells are resistant due to loss of TβRII expression [27]. In agreement with previous observations, we could demonstrate that TGF-β1 induced PAI-1 in a time-dependent manner in PANC-1 cells, but not in the other cell lines (Fig. 1). TGF-β1 also induced *βig-h3* mRNA expression in PANC-1 and CAPAN-1 cells in a time-dependent manner (Fig. 1). In contrast, TGF-β1 did not significantly alter *βig-h3* expression levels in the other three cell lines (data not shown).

The ability of TGF-β1 to induce *βig-h3* expression in CAPAN-1 cells that harbor Smad4 mutations [26] points to an Smad4-independent mechanism for this induction. Indeed, there is growing evidence that under some circumstances and in some cells, TGF-β signaling might be mediated in the absence of wild-type Smad4. Thus, in BxPc-3 pancreatic cancer cells, TGF-β1 exerts growth inhibitory effects despite the fact that this cell line has a deleted Smad4 gene [25]. In addition, in VACO-235 colon cells that harbor Smad4 mutations, TGF-β1 is still capable of exerting strong growth inhibitory effects [28]. These results collectively suggest that there is a non-Smad4-dependent pathway for TGF-β-mediated signaling.

In addition, our results that TGF-β1 induces *βig-h3* expression but not PAI-1 expression in CAPAN-1, whereas in PANC-1 TGF-β1 induces the expression of both genes, points to a dissociation of the TGF-β signaling pathway. This observation is in agreement with previous reports demonstrating that the expression of a truncated TβRII or a mutant TβRI does not attenuate TGF-β1-mediated induction of PAI-I, but does render Mv1Lu cells resistant to the antiproliferative effects of TGF-β1 [29]. In addition, transient overexpression of a mutated Smad3 protein blocks the antiproliferative effects of TGF-β1 in Mv1Lu cells, while still allowing for activation of PAI-I transcription [30]. Similarly, in pancreatic cancer cells, overexpression of Smad6 or Smad7 renders these cells insensitive to the growth inhibitory effects of TGF-β1, while still allowing for the induction of PAI-1 [23,24]. Collectively, these data suggest that there are divergent pathways of TGF-β signaling.

Since we observed TGF-β1-induced *βig-h3* expression in cultured pancreatic cancer cell lines, we next examined whether *βig-h3* is expressed in human pancreatic cancers in vivo. Therefore, 11 normal human pancreas samples and 27 human pancreatic cancer samples were investigated by Northern blot analysis. The *βig-h3* mRNA transcript was detected at moderate to strong levels in almost all pancreatic cancer samples, but was found at weak levels in normal pancreas samples. Densitometirc analysis indicated that there was a 32.4-fold increase in *βig-h3* mRNA levels in pancreatic cancer tissues in comparison to normal controls

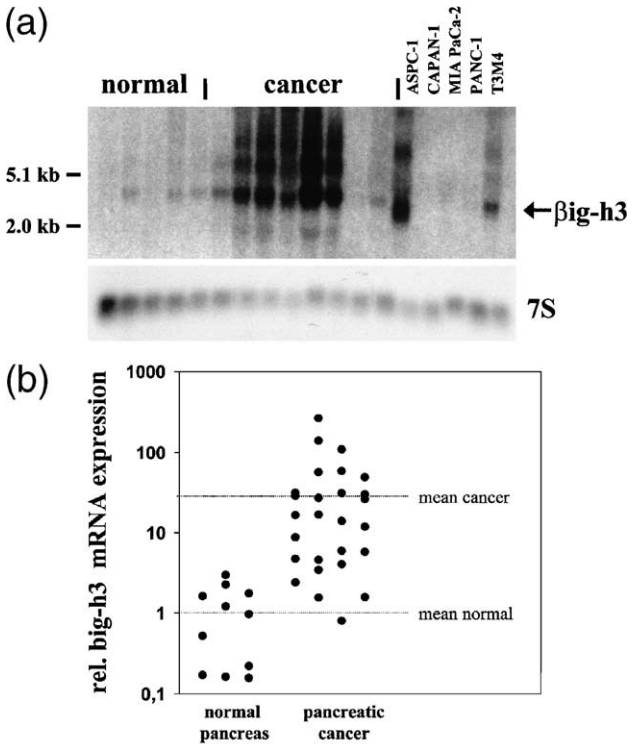

Fig. 2. Northern blot analysis of *βig-h3* mRNA in human pancreatic tissues. (a) Total RNA (20 μg) isolated from normal and cancerous pancreases and five pancreatic cancer cell lines was subjected to Northern blot analysis and probed with the ^{32}P labeled *βig-h3* cDNA. The blot was subsequently rehybridized with a 7S cDNA probe to verify equivalent RNA loading. (b) Densitometry of the Northern blots. Relative *βig-h3* mRNA expression was calculated as $OD_{\beta ig\text{-}h3}/OD_{7S}$ for each sample, and the fold increase over the mean in the normal pancreatic tissues was calculated. The mean in the normal samples was set to 1.

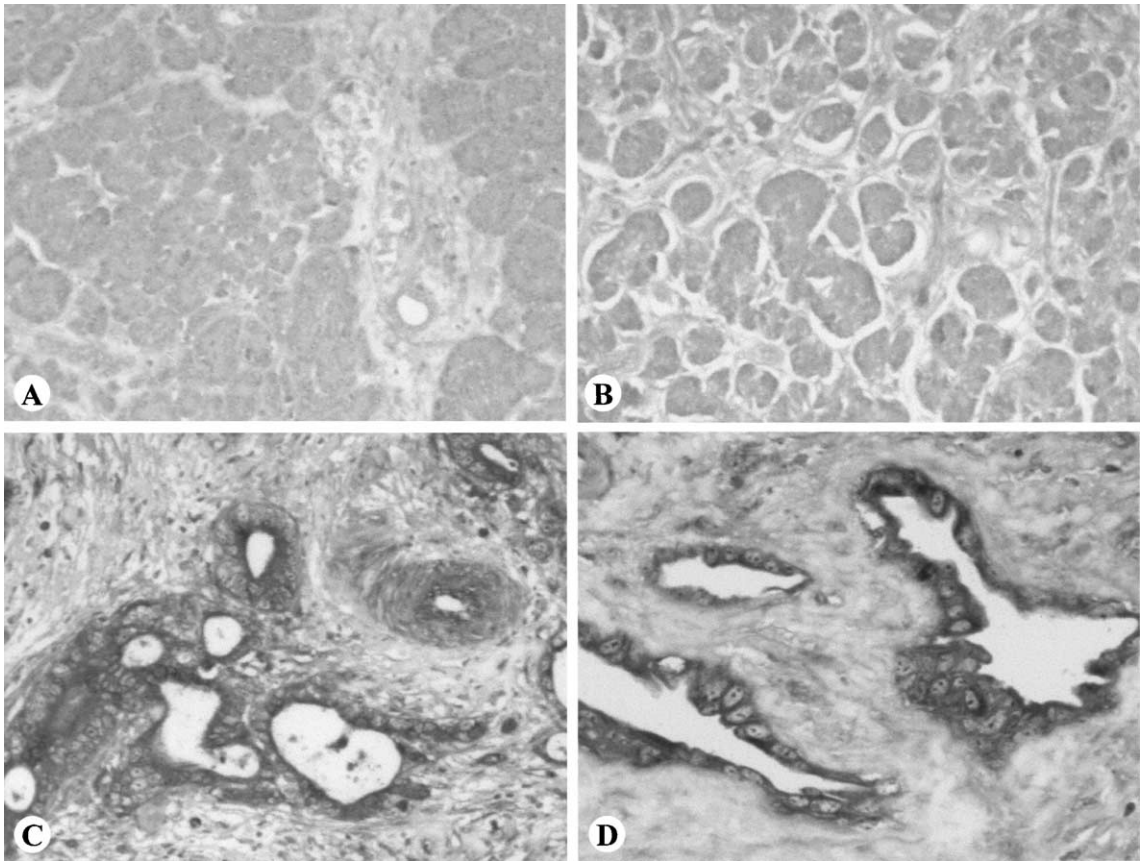

Fig. 3. In situ hybridization of *βig-h3* mRNA. (A) Normal pancreas: Weak signals were observed in some ductal and acinar cells. (B–D) Pancreatic cancer: Weak *βig-h3* mRNA signals were present in acinar cells that exhibited chronic pancreatitis-like alterations (B). *βig-h3* mRNA signals were predominantly observed in the cytoplasm of cancer cells.(C,D).

(Fig. 2b). Altogether, 26 of 27 pancreatic cancer samples expressed *βig-h3* levels that clearly exceeded the mean expression level in the normal samples.

To investigate the exact site of *βig-h3* mRNA expression in human pancreatic cancer samples, in situ hybridization was carried out next. The *βig-h3* mRNA in situ hybridization signal was intense in the cytoplasm of cancer cells (Fig. 3C,D). In contrast, in the normal pancreas, *βig-h3* mRNA signals were weakly present only in a few acinar and ductal cells (Fig. 3A,B). Previously, we have shown that human pancreatic cancers overexpress all three mammalian TGF-β isoforms [17]. Since our cell culture experiments indicate that TGF-β1 can up-regulate *βig-h3* even in the presence of Smad4 mutations, taken together, these observations suggest that up-regulation of *βig-h3* expression in vivo may be due, in part, to autocrine and paracrine effects of TGF-βs derived from the cancer cells.

The potential role of *βig-h3* in pancreatic cancer is not known. Since *βig-h3* is thought to be involved in cell attachment to the ECM, thereby influencing cell adhesion [2,31], it is possible that increased levels of *βig-h3* in pancreatic cancers influence cell adhesion and invasion of pancreatic cancer cells in vivo. Although further functional studies are required to address this question, the observation that *βig-h3* is strongly induced by TGF-β1 in some pancreatic cancer cells, irrespective of their Smad4 mutation status, and that *βig-h3* is overexpressed in pancreatic cancer cells, points to a potentially important autocrine loop that has the potential to contribute to the pathobiology of this disease.

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